

RECEPTOR BASED ANTAGONISTS AND
METHODS OF MAKING AND USING

This application claims priority of U.S. Provisional application No. 60/101,858
5 filed September 25, 1998. Throughout this application various publications
are referenced. The disclosures of these publications in their entireties are
hereby incorporated by reference into this application.

BACKGROUND OF THE INVENTION

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Although discovered for varying biological activities, ciliary neurotrophic
factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and
interleukin-6 (IL-6) comprise a defined family of cytokines (referred to herein
as the "CNTF family" of cytokines). These cytokines are grouped together
15 because of their distant structural similarities [Bazan, J. Neuron 7: 197-208
(1991); Rose and Bruce, Proc. Natl. Acad. Sci. USA 88: 8641-8645 (1991)], and,
perhaps more importantly, because they share " β " signal-transducing receptor
components [Baumann, et al., J. Biol. Chem. 265:19853-19862 (1993); Davis, et
al., Science 260: 1805-1808 (1993); Gearing et al., Science 255:1434-1437 (1992); Ip
20 et al., Cell 69: 1121-1132 (1992); Stahl, et al., J. Biol. Chem. 268: 7628-7631 (1993);
Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Receptor activation by this
family of cytokines results from either homo- or hetero-dimerization of these
 β components [Davis, et al. Science 260: 1805-1808 (1993), Murakami, et al.,
Science 260: 1808-1810 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)].
25 IL-6 receptor activation requires homodimerization of gp130 [Murakami, et al.
Science 260: 1808-1810 (1993), Hibi, et al., Cell 63: 1149-1157 (1990)], a protein
initially identified as the IL-6 signal transducer [Hibi, et al., Cell 63: 1149-1157

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(1990)]. CNTF, LIF and OSM receptor activation results from heterodimerization between gp130 and a second gp130-related protein known as LIFR β [Davis, et al., Science 260: 1805-1808 (1993)], that was initially identified by its ability to bind LIF [Gearing et al., EMBO J. 10: 2839-2848 (1991)].

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In addition to the β components, some of these cytokines also require specificity-determining " α " components that are more limited in their tissue distribution than the β components, and thus determine the cellular targets of the particular cytokines [Stahl and Yancopoulos, Cell 74: 587-590 (1993)].

10 Thus, LIF and OSM are broadly acting factors that may only require the presence of gp130 and LIFR β on responding cells, while CNTF requires CNTFR α [Stahl and Yancopoulos, Cell 74: 587-590 (1993)] and IL-6 requires IL-6R α [Kishimoto, et al., Science 258: 593-597 (1992)]. Both CNTFR α (Davis et al., Science 259:1736-1739 (1993) and IL-6R α [Hibi, et al. Cell 63:1149-1157, 15 Murakami, et al., Science 260:1808-1810 (1990); Taga, et al., Cell 58:573-581 (1989)] can function as soluble proteins, consistent with the notion that they do not interact with intracellular signaling molecules but that they serve to help their ligands interact with the appropriate signal transducing β subunits [Stahl and Yancopoulos, Cell 74: 587-590 (1993)].

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Additional evidence from other cytokine systems also supports the notion that dimerization provides a common mechanism by which all cytokine receptors initiate signal transduction. Growth hormone (GH) serves as perhaps the best example in this regard. Crystallographic studies have 25 revealed that each GH molecule contains two distinct receptor binding sites, both of which are recognized by the same binding domain in the receptor, allowing a single molecule of GH to engage two receptor molecules [de Vos, et

al., Science 255: 306-312 (1992)]. Dimerization occurs sequentially, with site 1 on the GH first binding to one receptor molecule, followed by the binding of site 2 to a second receptor molecule [Fuh, et al., Science 256: 1677-1680 (1992)]. Studies with the erythropoietin (EPO) receptor are also consistent with the importance of dimerization in receptor activation, as EPO receptors can be constitutively activated by a single amino acid change that introduces a cysteine residue and results in disulfide-linked homodimers [Watowich, et al., Proc. Natl. Acad. Sci. USA 89:2140-2144 (1992)].

10 In addition to homo- or hetero-dimerization of β subunits as the critical step for receptor activation, a second important feature is that formation of the final receptor complex by the CNTF family of cytokines occurs through a mechanism whereby the ligand successively binds to receptor components in an ordered manner [Davis, et al. Science 260:1805-1818 (1993); Stahl and

15 Yancopoulos, Cell 74: 587-590 (1993)]. Thus CNTF first binds to CNTFR α , forming a complex which then binds gp130 to form an intermediate (called here the $\alpha\beta 1$ intermediate) that is not signaling competent because it has only a single β component, before finally recruiting LIFR β to form a heterodimer of β components which then initiates signal transduction. Although a

20 similar intermediate containing IL-6 bound to IL-6R α and a single molecule of gp130 has not been directly isolated, we have postulated that it does exist by analogy to its distant relative, CNTF, as well as the fact that the final active IL-6 receptor complex recruits two gp130 monomers. Altogether, these findings led to a proposal for the structure of a generic cytokine receptor complex

25 (Figure 1) in which each cytokine can have up to 3 receptor binding sites: a site that binds to an optional α specificity-determining component (α site), a site that binds to the first β signal-transducing component ($\beta 1$ site), and a site

that binds to the second β signal-transducing component ($\beta 2$ site) [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. These 3 sites are used in sequential fashion, with the last step in complex formation -- resulting in β component dimerization -- critical for initiating signal transduction [Davis, et al. Science 5 260:1805-1818 (1993)]. Knowledge of the details of receptor activation and the existence of the non-functional $\beta 1$ intermediate for CNTF has led to the finding that CNTF is a high affinity antagonist for IL-6 under certain circumstances, and provides the strategic basis for designing ligand or receptor-based antagonists for the CNTF family of cytokines as detailed below.

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Once cytokine binding induces receptor complex formation, the dimerization of β components activates intracellular tyrosine kinase activity that results in phosphorylation of a wide variety of substrates [Ip, et al. Cell 69:121-1132 (1992)]. This activation of tyrosine kinase appears to be critical for 15 downstream events since inhibitors that block the tyrosine phosphorylations also prevent later events such as gene inductions [Ip, et al., Cell 69:121-1132 (1992); Nakajima and Wall, Mol. Cell. Biol. 11:1409-1418 (1991)]. Recently, we have demonstrated that a newly discovered family of non-receptor tyrosine kinases that includes Jak1, Jak2, and Tyk2 (referred to as the Jak/Tyk kinases) 20 [Firmbach-Kraft, et al., Oncogene 5:1329-1336 (1990); Wilks, et al., Mol. Cell. Biol. 11: 2057-2065 (1991)] and that are involved in signal transduction with other cytokines [Argetsinger, et al., Cell 74:237-244 (1993); Silvennoinen, et al., Proc. Natl. Acad. Sci. USA 90:8429-8433 (1993); Velazquez, et al., Cell 70: 313-322 (1992); Witthuhn, et al., Cell 74:227-236 (1993)], preassociate with the 25 cytoplasmic domains of the β subunits gp130 and LIFR β in the absence of ligand, and become tyrosine phosphorylated and activated upon ligand addition [Stahl et al., Science 263:92-95 (1994)]. Therefore these kinases appear

to be the most proximal step of intracellular signal transduction activated inside the cell as a result of ligand binding outside of the cell. Assay systems for screening collections of small molecules for specific agonist or antagonist activities based on this system are described below.

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The CNTF family of cytokines play important roles in a wide variety of physiological processes that provide potential therapeutic applications for both antagonists and agonists.

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SUMMARY OF THE INVENTION

An object of the present invention is the production of cytokine antagonists that are useful in the treatment of cytokine related diseases or disorders.

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Another object of the invention is the use of the disclosed cytokine antagonists for the treatment of cytokine related diseases or disorders. For example, an IL-6 antagonist described herein may be used for the treatment of osteoporosis, the primary and second effects of cancers, including multiple myeloma, or cachexia.

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Another object of the invention is the development of screening systems useful for identifying novel agonists and antagonists of cytokine receptors.

Another object of the invention is the development of screening systems useful for identifying small molecules that act as agonists or antagonists of

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the cytokines.

Another object of the invention is the development of screening systems

useful for identifying novel agonists and antagonists of members of the CNTF family of cytokines.

Another object of the invention is the development of screening systems
5 useful for identifying small molecules that act as agonists or antagonists of the CNTF family of cytokines.

BRIEF DESCRIPTION OF THE FIGURES

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FIGURE 1: Ordered binding of receptor components in a model of a generic cytokine receptor. The model indicates that cytokines contain up to 3 receptor binding sites and interact with their receptor components by binding first the optional α component, followed by binding to $\beta 1$, and then $\beta 2$. The β
15 components for many cytokine receptors interact through membrane proximal regions (shaded boxes) with the Jak/Tyk family of cytoplasmic protein tyrosine kinases. Only upon dimerization of β components is signal transduction initiated, as schematized by the tyrosine phosphorylations (P) of the β components and the Jak/Tyk kinases.

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FIGURE 2: CNTF inhibits IL-6 responses in a PC12 cell line (called PC12D) that expresses IL6R α , gp130, CNTFR α , but not LIFR β . Serum-deprived PC12D cells were incubated + IL-6 (50 ng/mL) in the presence or absence of CNTF as indicated. Some plates also received soluble IL6R α (1 mg/mL) or
25 soluble CNTFR α (1 mg/mL) as indicated. Cell lysates were subjected to immunoprecipitation with anti-gp130 and immunoblotted with anti-phosphotyrosine. Tyrosine phosphorylation of gp130 is indicative of IL-6

C2
cont.

Cly bridge is shown in bold type (amino acids 359, 360). Amino acids 361 to 592 are from the Fc domain of human IgG1 (Lewis et al., J. Immunol. 151:2829-2838 (1993). (+) mark the two cysteines (amino acids number 371 and 374) of the IgG hinge preceding the Fc that form the inter-chain disulfide bridges that link two Fc domains. (•) shows the position of the STOP codon.

FIGURE 6: The CNTF/IL-6/IL-11 receptor system. The ordered formation of the hexameric signal transducing receptor complex is depicted schematically. The cytokine associates with the $R\alpha$ component to form an obligatory cytokine• $R\alpha$ complex (K_d is about 5 nM). This low affinity complex next associates with the first signal transducing component, marked $\beta 1$, to form a high affinity cytokine• $R\alpha$ • $\beta 1$ complex (K_d is about 10 pM). In the case of IL-6 $R\alpha$, this component is gp130. This trimeric high affinity complex subsequently associates with another such complex. Formation of this complex results in signal transduction as it involves dimerization of two signal transducing components, marked $\beta 1$ and $\beta 2$ respectively (adapted from (Ward et al., J. Bio. Chem. 269:23286-23289 (1994); Stahl and Yancopoulos, J. Neurobiology 25:1454-1466 (1994); Stahl and Yancopoulos, Cell 74:587-590 (1993).

FIGURE 7: Design of heterodimeric receptor-based ligand traps for IL-6. The heterodimeric ligand trap is comprised of two interdisulfide linked proteins, gp130-Fc and IL-6 $R\alpha$ -Fc. The gp130-Fc•IL-6 $R\alpha$ -Fc complex (upper panel) is shown to mimic the high affinity cytokine• $R\alpha$ • $\beta 1$ complex (lower panel). The ligand trap functions as an antagonist by sequestering IL-6 and thus rendering unavailable to interact with the native receptors on IL-6-responsive cells.

FIGURE 8. Heteromeric immunoglobulin Heavy/Light Chain Receptor Fusions. An example of a heavy/light chain receptor fusion molecule is schematically depicted. The extracellular domain of gp130 is fused to C γ ,

5 whereas the extracellular domain of IL-6R α is fused to the constant region of the kappa chain (κ). The inter-chain disulfide bridges are also depicted (S-S).

C3> ~~FIGURE 9. Amino acid sequence of gp130-C γ 1. Key: Amino acids 1 to 619 are from human gp130 (Hibi, et al., Cell 63:1149-1157 (1990). Ser-Gly bridge is shown in bold type. Amino acids 662 to 651 are from the constant region of human IgG1 (Lewis et al., J. Immunol. 151:2829-2838 (1993). (*) shows the position of the STOP codon.~~

C4> ~~FIGURE 10. Amino acid sequence of gp130 Δ 3fibro. Key: Amino acids 1 to 330 are from human gp130 (Hibi et al., Cell 63:1149-1157 (1990). Other symbols as described in Figure 9.~~

C5> ~~FIGURE 11. Amino acid sequence of J-CH1. Key: The Ser-Gly bridge is shown in bold, the J-peptide is shown in italics, the CH1 domain is underlined.~~

C6> ~~FIGURE 12. Amino acid sequence of C γ 4. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 239 comprise the C γ 4 sequence.~~

C7> 25 ~~FIGURE 13. Amino acid sequence of κ -domain. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 108 comprise the κ domain. The C-terminal cysteine (amino acid 108) is that involved in the disulfide bond of the κ -domain with the CH1 domain of C γ .~~

C8) ~~FIGURE 14. Amino acid sequence of λ domain. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 106 comprise the λ domain (Cheung, et al., J. Virol. 66: 6714-6720 (1992). The C-terminal cysteine (amino acid 106) is that involved in the disulfide bond of the λ domain with the CH1 domain of C γ .~~

C9) ~~FIGURE 15. Amino acid sequence of the soluble IL-6R α domain. Key: Amino acids 1 to 358 comprise the soluble IL-6R α domain (Yamasaki, et al., Science 241:825-828 (1988). The Ala-Gly bridge is shown in bold type.~~

C10) ~~FIGURE 16. Amino acid sequence of the soluble IL-6R α 313 domain. Key: Amino acids 1 to 313 comprise the truncated IL-6R α domain (IL-6R α 313). The Thr-Gly bridge is shown in bold type.~~

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FIGURE 17: Purification of gp130-C γ 1•IL-6R α - κ . 4% to 12% SDS-PAGE gradient gel run under non-reducing conditions. Proteins were visualized by staining with silver. Lane 1: approximately 100 ng of material purified over Protein A Sepharose (Pharmacia). Lane 2: Molecular size standards (Amersham). Lane 3: The Protein A-purified material shown here after further purification over an IL-6 affinity chromatography step. The positions of the gp130-C γ 1 dimer [(gp130-C γ 1) $_2$], the gp130-C γ 1 dimer associated with one IL-6R α - κ [(gp130-C γ 1) $_2$ •(IL-6R α - κ) $_1$], and the gp130-C γ 1 dimer associated with two IL-6R α - κ [(gp130-C γ 1) $_2$ •(IL-6R α - κ) $_2$] are shown, as well as the sizes for the molecular size standards in kilodaltons (200, 100, and 46).

FIGURE 18. IL-6 dissociates slowly from the ligand trap. The dissociation

Year	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100
1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	

 $\alpha| \rangle$

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C12)

C/3)

C/3
cont

~~sequence of fusion polypeptide designated 603 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.~~

C14)

~~FIGURES 23A-23D - Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 622 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.~~

C15)

~~FIGURE 24A-24F - Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 412 which is capable of binding the cytokine IL-6 to form a nonfunctional complex~~

C16)

~~FIGURE 25A-25F - Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 616 which is capable of binding the cytokine IL-6 to form a nonfunctional complex.~~

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C17

~~FIGURE 26A-26E - Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 569 which is capable of binding the cytokine IL-1 to form a nonfunctional complex.~~

FIGURE 27 - shows that an IL-4 trap designated 4SC375, which is a fusion polypeptide of IL-2R γ -scb-IL4R α -Fc Δ C1, is several orders of magnitude better as an IL-4 antagonist than IL4R α Fc Δ C1 alone in the TF1 cell bioassay.

FIGURE 28/- shows that an IL-4 trap designated 4SC375 displays antagonistic activity in the TF1 cell bioassay equivalent to an IL-4 trap designated 4SC424 (described in Figs. 21A-21D) which is a fusion polypeptide of IL-2R γ -IL4R α -Fc Δ C1 having the IL-2R γ component flush with the IL-4R α component.

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FIGURE 29 - shows that the IL6 trap (6SC412 IL6R-scb-gpx-FcΔC1) described in Figs. 24A-24F is a better antagonist of IL-6 in the XG1 bioassay than the neutralizing monoclonal antibody to human IL-6 - BE8.

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FIGURE 30 - shows that the trap 1SC569 (described in Figs. 26A-26E) is able to antagonize the effects of IL-1 and block the IL-6 production from MRC 5 cells upon treatment with IL-1.

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~~FIGURE 31A-31G - The nucleotide and encoded amino acid sequence of the IL-4Rα:IL-13Rα1.Fc single chain trap construct is set forth.~~

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~~FIGURE 32A-32G - The nucleotide and encoded amino acid sequence of the IL-13Rα1:IL-4Rα.Fc single chain trap construct is set forth.~~

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising:

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a) a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor;

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b) a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor; and

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c) a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

By "cytokine binding portion" what is meant is the minimal portion of the extracellular domain necessary to bind the cytokine. It is accepted by those of skill in the art that a defining characteristic of a cytokine receptor is the presence of the two fibronectin-like domains that contain canonical cysteines and of the WSXWS box (Bazan, J.F., 1990, PNAS 87: 6934-6938). Sequences encoding the extracellular domains of the binding component of the cytokine's receptor and of the signal transducing component of the cytokine's receptor may also be used to create the fusion polypeptide of the invention. Similarly, longer sequences encoding larger portions of the components of the cytokine's receptor may be used. However, it is contemplated that fragments smaller than the extracellular domain will function to bind the cytokine and therefore, the invention contemplates fusion polypeptides comprising the minimal portion of the extracellular domain necessary to bind the cytokine as the cytokine binding portion.

The invention comprises a "specificity determining component" of a cytokine's receptor and a "signal transducing component" of the cytokine's receptor. Regardless of the nomenclature used to designate a particular component or subunit of a cytokine receptor, one skilled in the art would recognize which component or subunit of a receptor is responsible for determining the cellular target of the cytokine, and thus would know which component constitutes the "specificity determining component."

Similarly, regardless of the nomenclature used, one of skill in the art would know which component or subunit of a receptor would constitute the "signal transducing component." As used herein, the "signal transducing component" is a component of the native receptor which is not the specificity determining component and which, does not bind or weakly binds the cytokine in the absence of the specificity determining component. In the

native receptor, the "signal transducing component" may participate in signalling.

For example, while some cytokine receptors have components designated α and β , the IL-4 receptor has a signal transducing component referred to as IL-2R γ . However, regardless of what name is associated with that component, one skilled in the art would know which component of the IL-4 receptor is the signal transducing component. Thus to practice the present invention and create a high affinity trap for IL-4, one of skill in the art would create an isolated nucleic acid comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the IL-4 receptor (IL-4R α); a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the IL-4 receptor (IL-2R γ); and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component (for example, an Fc domain of IgG) to create a high affinity trap for IL-4.

Some further examples of the receptor components that may be used to prepare cytokine antagonists according to the invention are set forth in Table 1. The Table 1 sets forth, by way of example but not by way of limitation, some of the varied nomenclature used in the scientific literature to describe those components which function as specificity determining components and those which function as signal transducing components of certain cytokine receptors.

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TABLE 1

<u>Cytokine</u>	<u>Specificity determining Component</u>	<u>Signal transducing Component</u>
Interleukin-1 (IL-1)	Type I IL-1R (ref. 8) Type II IL-1R (ref. 8) IL-1RI (ref. 11) IL-1RII (ref. 11)	IL-1R AcP (refs. 8, 11)
Interleukin-2 (IL-2)	α -subunit (ref. 2) α -chain (ref. 3) IL-2R α (ref. 1)	β -chain (ref. 3) β -subunit (ref. 2) γ -chain (ref. 3) IL-2R β (refs. 1, 10) IL-2R γ (refs. 1, 10)
Interleukin-3 (IL-3)	IL-3R α (ref. 1) α -subunit (ref. 2) α -receptor component (ref. 5)	β_c (ref. 1) β -subunit (ref. 2) β -chain (ref. 3) β -receptor component (ref. 5)
Interleukin-4 (IL-4)	IL-4R (ref. 1)	γ -chain (ref. 3) IL-2R γ (ref. 1)
Interleukin-5 (IL-5)	IL-5R α (ref. 1) α -subunit (ref. 2) α -receptor component (ref. 5)	β_c (ref. 1) β -subunit (ref. 2) β -chain (ref. 3) β -receptor component (ref. 5)

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TABLE 1 (CONT'D)

<u>Cytokine</u>	<u>Specificity determining Component</u>	<u>Signal transducing Component</u>
Granulocyte macrophage-colony stimulating factor (GM-CSF)	α -receptor component (ref. 5) α -subunit (ref. 2) GMR α (refs. 1, 2)	β -receptor component (ref. 5) β -subunit (ref. 2) β -chain (ref. 3) β_c (ref. 1) GMR β (refs. 1, 2)
Leukemia inhibitory factor (LIF)	LIFBP (ref. 1) α -receptor component (ref. 5)	gp130 (refs. 1, 3) β -receptor component (ref. 5)
Interleukin-11 (IL-11)	α -chain (ref. 4) NR1 (ref. 4)	gp130 (ref. 4)
Interleukin-15 (IL-15)	IL-15R α (ref. 10)	IL-2R β (ref. 10) IL-2R γ (ref. 10)
Interferon- γ (IFN γ)	IFN- γ R (ref. 7) IFN- γ R1 (ref. 7)	AF-1 (ref. 7) IFN- γ R2 (ref. 7)
TGF β	Type II (refs. 6, 9)	Type I (refs. 6, 9)

Only a few of the multitude of references are cited in Table 1, and they are set forth as follows:

1. Sato and Miyajima, Current Opinions in Cell Biology 6: 174-179 (1994) -
5 See page 176, lines 9-16;
2. Miyajima, et al., Annual Review of Immunology 10: 295-331 (1992) -
See page 295, line 4 to page 296, line 1; page 305, last paragraph;
3. Kondo, et al, Science 262: 1874-1877 (1993) - See page 1874, cols. 1 & 2;
4. Hilton, et al, EMBO Journal 13: 4765-4775 (1994) - See page 4766, col. 1,
10 lines 20-24;
5. Stahl and Yancopoulos, Cell 74: 587-590 (1993) - See page 587, column 2,
lines 15-22;
6. Bassing, et al, Journal of Biological Chemistry 269: 14861-14864 (1994) -
See page 14861, col. 2, lines 1-9 and 21-28;
- 15 7. Kotenko, et al, Journal of Biological Science 270: 20915-20921 (1995) - See
page 20915, lines 1-5 of the abstract;
8. Greenfeder, et al., Journal of Biological Chemistry 270: 13757-13765
(1995) - See page 13757, col. 1, line 6 to col. 2, line 3 and col. 2, lines 10-12; page
13764, col. 2, last 3 lines and page 13765, col. 1, lines 1-7;
- 20 9. Lebrun and Vale, Molecular Cell Biology 17: 1682-1691 (1997) - See page
1682, Abstract lines 2-6;
10. Kennedy and Park, Journal of Clinical Immunology 16: 134-143 (1996) -
See page 134, lines 1-7 of the abstract; page 136, col 2., lines 1-5;
11. Wesche, et al., Journal of Biological Chemistry 272: 7727-7731 (1997) See
25 page 7731, lines 20-26.

Kotenko, et al. recently identified the IL-10R2 (IL-10R β) chain which is reported to serve as an accessory chain that is essential for the active IL-10 receptor complex and for initiating IL-10 induced signal transduction events
30 (S.V. Kotenko, et al., The EMBO Journal, 1997, Vol. 16: 5894-5903). Additional

cytokines and their receptors are described in Appendix II, page A:9 of Immunobiology, The Immune System In Health and Disease, 2nd Edition, by Charles A. Janeway, Jr. and Paul Travers, published by Current Biology Ltd./Garland Publishing Inc., copyright 1996.

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In preparing the nucleic acid sequence encoding the fusion polypeptide of the invention, the first, second, and third components of the fusion polypeptide are encoded in a single strand of nucleotides which, when expressed by a host vector system, produces a monomeric species of the fusion polypeptide. The monomers thus expressed then multimerize due to the interactions between the multimerizing components (the third fusion polypeptide components). Producing the fusion polypeptides in this manner avoids the need for purification of heterodimeric mixtures that would result if the first and second components were produced as separate molecules and then multimerized. For example, U.S. Patent No. 5,470,952 issued November 28, 1995 describes the production of heterodimeric proteins that function as CNTF or IL-6 antagonists. The heterodimers are purified from cell lines cotransfected with the appropriate alpha and beta components. Heterodimers are then separated from homodimers using methods such as passive elution from preparative, nondenaturing polyacrylamide gels or by using high pressure cation exchange chromatography. The need for this purification step is avoided by the methods of the present invention.

In addition, PCT International Application WO 96/11213 published 18 April 1996 entitled Dimeric IL-4 Inhibitors states that the applicant has prepared homodimers in which two IL-4 receptors are bound by a polymeric spacer and has prepared heterodimers in which an IL-4 receptor is linked by a polymeric spacer to an IL-2 receptor gamma chain. The polymeric spacer described is polyethylene glycol (PEG). The two receptor components, IL-4R and IL-2Rgamma are separately expressed and purified. Pegylated homodimers and heterodimers are then produced by joining the components together using bi-

functional PEG reagents. It is an advantage of the present invention that it avoids the need for such time consuming and costly purification and pegylation steps.

5 In one embodiment of the invention, the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component. In another embodiment of the invention, the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component. Further embodiments of the invention may be prepared in which the order of the first, second and third fusion polypeptide components is rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5' to 3' may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

In further embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the hematopoietin family of cytokines selected from the group consisting of interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-11, interleukin-13, interleukin-15, granulocyte macrophage colony stimulating factor, oncostatin M, and leukemia inhibitory factor.

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In additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the interferon family of cytokines selected from the group consisting of IFN-gamma, IFN-alpha, and IFN-beta.

In additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the immunoglobulin superfamily of cytokines selected from the group consisting of B7.1 (CD80) and B7.2 (B70).

5 In still further embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the TNF family of cytokines selected from the group consisting of TNF-alpha, TNF-beta, LT-beta, CD40 ligand, Fas ligand, CD 27 ligand, CD 30 ligand, and 4-1BBL.

10 In additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a cytokine selected from the group consisting of interleukin-1, interleukin-10, interleukin-12, interleukin-14, interleukin-18, and MIF.

15 Because specificity determination and signal transduction occurs by a similar mechanism in the TGF- β /BMP family of cytokines (See D. Kingsley, Genes & Development, 1994, 8: 133-146; J. Wrana, Miner Electrolyte Metab, 24: 120-130 (1998); R. Derynck and X. Feng, Biochimica et Biophysica Acta 1333 (1997) F105-F150; and J. Massague and F. Weis-Garcia, "Serine/threonine Kinase

20 Receptors: Mediators of Transforming Growth Factor Beta Family Signals" In Cancer Surveys, Vol. 27: Cell Signalling, 1996, Imperial Cancer Research Fund) the present invention may be used to produce high affinity antagonists for cytokines that are members of the TGF- β /BMP family.

25 Therefore, in additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the TGF- β /BMP family selected from the group consisting of TGF- β 1, TGF- β 2, TGF- β 3, BMP-2, BMP-3a, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8a, BMP-8b, BMP-9, BMP-10, BMP-11, BMP-15, BMP-16, endometrial bleeding associated factor (EBAF), growth

30 differentiation factor-1 (GDF-1), GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8,

GDF-9, GDF-12, GDF-14, mullerian inhibiting substance (MIS), activin-1, activin-2, activin-3, activin-4, and activin-5.

In alternative embodiments of the invention, the specificity determining component, the signal transducing component, or both, may be substituted for by a single chain Fv. A single chain Fv (scFv) is a truncated Fab having only the V region of a heavy chain linked by a stretch of synthetic peptide to a V region of a light chain. See, for example, US Patent Nos. 5,565,332; 5,733,743; 5,837,242; 5,858,657; and 5,871,907 assigned to Cambridge Antibody Technology Limited incorporated by reference herein. Thus the present invention contemplates, for example, an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor; a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of an scFv capable of binding the cytokine at a site different from the site at which the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor binds; and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component. Alternatively, the specificity determining component may be substituted for by a scFv that binds to a site on the cytokine different from the site at which the signal transducing component binds. Thus the invention contemplates an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of a scFv that binds to a site on the cytokine different from the site at which the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor binds;

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a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor; and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

In another embodiment, the invention contemplates an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising: a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of a first scFv that binds to a site on the cytokine; a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence a second scFv that binds to a site on the cytokine different from the site at which the first scFv binds; and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

In all of the above described embodiments comprising scFv's, the invention also contemplates embodiments in which the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component; embodiments in which the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component; and further embodiments of the invention in which the order of the first, second and third fusion polypeptide components is rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5' to 3' may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

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In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(Δ C1). Alternatively, the multimerizing component may be an Fc domain in which a cysteine within the first five amino acids has been substituted for by another amino acid such as, for example, serine or alanine.

The present invention also provides for fusion polypeptides encoded by the isolated nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the third multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the third multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the

expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS, CHO, 293, BHK or NS0 cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The present invention provides novel antagonists which are based on receptor components that are shared by cytokines such as the CNTF family of cytokines.

The invention described herein contemplates the production of antagonists to any cytokine that utilizes an α specificity determining component which, when combined with the cytokine, binds to a first β signal transducing component to form a nonfunctional intermediate which then binds to a second β signal transducing component causing β - receptor dimerization and consequent signal transduction. According to the invention, the soluble α specificity determining component of the receptor ($sR\alpha$) and the extracellular domain of the first β signal transducing component of the cytokine receptor ($\beta 1$) are combined to form heterodimers ($sR\alpha:\beta 1$) that act as antagonists to the cytokine by binding the cytokine to form a nonfunctional complex.

As described in Example 1, CNTF and IL-6 share the $\beta 1$ receptor component gp130. The fact that CNTF forms an intermediate with CNTF α and gp130

can be demonstrated (Example 1) in cells lacking LIFR β , where the complex of CNTF and CNTFR α binds gp130, and prevents homodimerization of gp130 by IL-6 and IL-6R α , thereby blocking signal transduction. These studies provide the basis for the development of the IL-6 antagonists described

5 herein, as they show that if, in the presence of a ligand, a nonfunctional intermediate complex, consisting of the ligand, its α receptor component and its β 1 receptor component, can be formed, it will effectively block the action of the ligand. Other cytokines may use other β 1 receptor components, such as LIFR β , which may also be used to produce antagonists according to the
10 present invention.

Thus for example, in one embodiment of the invention, effective antagonists of IL-6 or CNTF consist of heterodimers of the extracellular domains of the α specificity determining components of their receptors (sIL-6R α and sCNTFR α respectively) and the extracellular domain of gp130. The resultant
15 heterodimers, which are referred to hereinafter as sIL-6R α : β 1 and sCNTFR α : β 1 respectively, function as high-affinity traps for IL-6 or CNTF, respectively, thus rendering the cytokine inaccessible to form a signal transducing complex with the native membrane-bound forms of their
20 receptors.

Although soluble ligand binding domains from the extracellular portion of receptors have proven to be somewhat effective as traps for their ligands and thus act as antagonists [Bargetzi, et al., Cancer Res. 53:4010-4013 (1993); , et al.,
25 Proc. Natl. Acad. Sci. USA 89: 8616-8620 (1992); Mohler, et al., J. Immunol. 151: 1548-1561 (1993); Narazaki, et al., Blood 82: 1120-1126 (1993)], the IL-6 and CNTF receptors are unusual in that the α receptor components constitute

ligand binding domains that, in concert with their ligands, function effectively in soluble form as receptor agonists [Davis, et al. Science 259:1736-1739 (1993); Taga, et al., Cell 58: 573-581 (1989)]. The sR α : β 1 heterodimers prepared according to the present invention provide effective traps for their ligands, binding these ligands with affinities in the picomolar range (based on binding studies for CNTF to PC12D cells) without creating functional intermediates. The technology described herein may be applied to develop a cytokine trap for any cytokine that utilizes an α -component that confers specificity, as well as a β component which, when bound to the α -specificity component, has a higher affinity for the cytokine than either component alone. Accordingly, antagonists according to the invention include antagonists of interleukins 1 through 5 [IL-1, Greenfeder, et al. J. Biol. Chem. 270:13757-13765 (1995); Guo, et al. J. Biol. Chem. 270:27562-27568 (1995)], IL-2; [Taniguchi, et al. European Patent Nos. 0386289-A and 0386304-A (1990); Takeshita, et al. Science 257:379-382 (1992)]; IL-3; [Kitamura, et al. Cell 66:1165-1174 (1991)], IL-4; [Idzerda, et al. J. Exp. Med. 171:861-873 (1990)], IL-5; [Taverneir, et al. Cell 66:1175-1184 (1991)], IL-11 [(Cherel, et al. Direct Submission to EMBL/GenBank/DDBJ databases; accession No. Z38102)], interleukin 15 [IL-15; Hemar, et al. J. Cell Biol. 129:55-64 (1995); Taniguchi, et al. European Patent Nos. 0386289-A and 0386304-A (1990); Takeshita, et al. Science 257:379-382 (1992)], granulocyte-macrophage colony stimulating factor [GM-CSF; Hayashida, et al. Proc. Natl. Acad. Sci. U.S.A. 97:9655-9659 (1990)], LIF, gamma interferon [IFN γ ; Aguet, et al. Cell 55:273-280 (1988); Soh, et al. Cell 76:793-802 (1994)], and transforming growth factor beta [TGF β ; Inagaki, et al. Proc. Natl. Acad. Sci. USA 90:5359-5363 (1993)].

The α and β receptor extracellular domains may be prepared using methods

known to those skilled in the art. The CNTFR α receptor has been cloned, sequenced and expressed [Davis, et al. (1991) Science 253:59-63 which is incorporated by reference in its entirety herein]. The cloning of LIFR β and gp130 are described in Gearing et al. in EMBO J. 10:2839-2848 (1991), Hibi, et al. 5 Cell 63:1149-1157 (1990) and in published PCT application WO 93/10151 published May 27, 1993, all of which are incorporated by reference in their entirety herein.

The receptor molecules useful for practicing the present invention may be prepared by cloning and expression in a prokaryotic or eukaryotic 10 expression system. The recombinant receptor gene may be expressed and purified utilizing any number of methods. The gene encoding the factor may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The recombinant factors may be purified by any technique which allows 15 for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange 20 chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The sR α : β heterodimeric receptors may be engineered using known fusion regions, as described in published PCT application WO 93/10151 published May 27, 1993 entitled "Receptor for Oncostatin M and Leukemia 25 Inhibitory Factor" which describes production of β receptor heterodimers, or they may be prepared by crosslinking of extracellular domains by chemical means. The domains utilized may consist of the entire extracellular domain

of the α and β components, or they may consist of mutants or fragments thereof that maintain the ability to form a complex with its ligand and other components in the sR α : β 1 complex. For example, as described below in example 4, IL-6 antagonists have been prepared using gp130 that is lacking its
5 three fibronectin-like domains.

In one embodiment of the invention, the extracellular domains are engineered using leucine zippers. The leucine zipper domains of the human transcription factors c-jun and c-fos have been shown to form stable heterodimers [Busch and Sassone-Corsi, Trends Genetics 6: 36-40 (1990);
10 Gentz, et al., Science 243: 1695-1699 (1989)] with a 1:1 stoichiometry. Although jun-jun homodimers have also been shown to form, they are about 1000-fold less stable than jun-fos heterodimers. Fos-fos homodimers have not been detected.

The leucine zipper domain of either c-jun or c-fos are fused in frame at
15 the C-terminus of the soluble or extracellular domains of the above mentioned receptor components by genetically engineering chimeric genes. The fusions may be direct or they may employ a flexible linker domain, such as the hinge region of human IgG, or polypeptide linkers consisting of small amino acids such as glycine, serine, threonine or alanine, at various lengths
20 and combinations. Additionally, the chimeric proteins may be tagged by His-His-His-His-His (His6), [SEQ. ID NO. 1] to allow rapid purification by metal-chelate chromatography, and/or by epitopes to which antibodies are available, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

25 In another embodiment, as described below in Example 3. the sR α : β 1 heterodimer is prepared using a similar method, but using the Fc-domain of human IgG1 [Aruffo, et al., Cell 67:35-44 (1991)]. In contrast to the latter,

formation of heterodimers must be biochemically achieved, as chimeric molecules carrying the Fc-domain will be expressed as disulfide-linked homodimers. Thus, homodimers may be reduced under conditions that favor the disruption of inter-chain disulfides but do not effect intra-chain disulfides. Then monomers with different extracellular portions are mixed in equimolar amounts and oxidized to form a mixture of homo- and heterodimers. The components of this mixture are separated by chromatographic techniques. Alternatively, the formation of this type of heterodimers may be biased by genetically engineering and expressing molecules that consist of the soluble or extracellular portion of the receptor components followed by the Fc-domain of hIgG, followed by either the c-jun or the c-fos leucine zippers described above [Kostelny, et al., J. Immunol. 148: 1547-1553 (1992)]. Since these leucine zippers form predominately heterodimers, they may be used to drive formation of the heterodimers where desired. As for the chimeric proteins described using leucine zippers, these may also be tagged with metal chelates or an epitope. This tagged domain can be used for rapid purification by metal-chelate chromatography, and/or by antibodies, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

In additional embodiments, heterodimers may be prepared using other immunoglobulin derived domains that drive the formation of dimers. Such domains include, for example, the heavy chains of IgG (C γ 1 and C γ 4), as well as the constant regions of kappa (κ) and lambda (λ) light chains of human immunoglobulins. The heterodimerization of C γ with the light chain occurs between the CH1 domain of C γ and the constant region of the light chain (CL), and is stabilized by covalent linking of the two domains via a single disulfide bridge. Accordingly, as described in Example 4, constructs may be

prepared using these immunoglobulin domains. Alternatively, the immunoglobulin domains include domains that may be derived from T cell receptor components which drive dimerization,

In another embodiment of the invention the sR α : β 1 heterodimers are prepared by expression as chimeric molecules utilizing flexible linker loops. A DNA construct encoding the chimeric protein is designed such that it expresses two soluble or extracellular domains fused together in tandem ("head to head") by a flexible loop. This loop may be entirely artificial (e.g. polyglycine repeats interrupted by serine or threonine at a certain interval) or "borrowed" from naturally occurring proteins (e.g. the hinge region of hIgG). Molecules may be engineered in which the order of the soluble or extracellular domains fused is switched (e.g. sIL6R α /loop/sgp130 or sgp130/loop/sIL-6R α) and/or in which the length and composition of the loop is varied, to allow for selection of molecules with desired characteristics.

Alternatively, the heterodimers made according to the present invention may be purified from cell lines cotransfected with the appropriate α and β components. Heterodimers may be separated from homodimers using methods available to those skilled in the art. For example, limited quantities of heterodimers may be recovered by passive elution from preparative, nondenaturing polyacrylamide gels. Alternatively, heterodimers may be purified using high pressure cation exchange chromatography. Excellent purification has been obtained using a Mono S cation exchange column.

In addition to sR α : β 1 heterodimers that act as antagonists by binding free CNTF or IL-6, the present invention also contemplates the use of engineered, mutated versions of IL-6 with novel properties that allow it to bind to IL-6R α and a single gp130 molecule, but fail to engage the second

gp130 to complete β component homodimerization, and thus act as an effective IL-6 antagonist on any IL-6 responsive cell. Our model for the structure of the IL-6 and CNTF receptor complexes indicates that these cytokines have distinct sites for binding the α , $\beta 1$, and $\beta 2$ receptor

5 components [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Mutations of critical amino acid residues comprising each of these sites gives rise to novel molecules which have the desired antagonistic properties. Ablation of the $\beta 1$ site would give a molecule which could still bind to the α receptor component but not the $\beta 1$ component, and thereby comprise an antagonist
10 with nanomolar affinity. Mutations of critical amino acid residues comprising the $\beta 2$ site of IL-6 (IL-6 $\beta 2^-$) would give a molecule that would bind to IL-6R α and the first gp130 monomer, but fail to engage the second gp130 and thus be functionally inactive. Similarly, mutations of the CNTF $\beta 2$ site would give a molecule (CNTF $\beta 2^-$) that would bind CNTFR α and gp130,
15 but fail to engage LIFR β , thereby antagonizing CNTF action by forming the non-functional $\beta 1$ intermediate. Based on the binding results described above where CNTF forms the $\beta 1$ intermediate with high affinity, both CNTF $\beta 2^-$ and IL-6 $\beta 2^-$ would constitute antagonists with affinity in the range of 10 pM.

A variety of means are used to generate and identify mutations of IL-6
20 or CNTF that have the desired properties. Random mutagenesis by standard methods of the DNA encoding IL-6 or CNTF may be used, followed by analysis of the collection of products to identify mutated cytokines having the desired novel properties as outlined below. Mutagenesis by genetic engineering has been used extensively in order to elucidate the structural
25 organization of functional domains of recombinant proteins. Several different approaches have been described in the literature for carrying out deletion or substitution mutagenesis. The most successful appear to be

alanine scanning mutagenesis [Cunningham and Wells (1989), Science 244: 1081-1085] and homolog-scanning mutagenesis [Cunningham, et al., (1989), Science 243:1330-1336].

5 Targeted mutagenesis of the IL-6 or CNTF nucleic acid sequences using such methods can be used to generate CNTF β 2- or IL-6 β 2- candidates. The choice of regions appropriate for targeted mutagenesis is done systematically, or determined from studies whereby panels of monoclonal antibodies against each factor are used to map regions of the cytokine that might be exposed after binding of the cytokine to the α receptor component alone, or to the $\alpha\beta$ 1
10 heterodimeric soluble receptors described above. Similarly, chemical modification or limited proteolysis of the cytokine alone or in a complex bound to the α receptor component or the $\alpha\beta$ 1 heterodimeric soluble receptors described above, followed by analysis of the protected and exposed regions could reveal potential β 2 binding sites.

15 Assays for identifying CNTF or IL-6 mutants with the desired properties involve the ability to block with high affinity the action of IL-6 or CNTF on appropriately responsive cell lines [Davis, et al., Science 259: 1736-1739 (1993); Murakami, et al., Proc. Natl. Acad. Sci. USA 88: 11349-11353 (1991)]. Such assays include cell proliferation, survival, or DNA synthesis
20 driven by CNTF or IL-6, or the construction of cell lines where binding of factor induces production of reporters such as CAT or β -galactosidase [Savino, et al., Proc. Natl. Acad. Sci. USA 90: 4067-4071 (1993)].

Alternatively, the properties of various mutants may be assessed with a receptor-based assay. One such assay consists of screening mutants for their
25 ability to bind the sR α : β 1 receptor heterodimers described above using epitope-tagged [Davis et al., Science 253: 59-63 (1991)] sR α : β 1 reagents. Furthermore, one can probe for the presence or absence of the β 2 site by

assessing whether an epitope-tagged soluble $\beta 2$ reagent will bind to the cytokine in the presence of the $\beta 1$ heterodimer. For example, CNTF only binds to LIFR β (the $\beta 2$ component) in the presence of both CNTFR α and gp130 [Davis, et al. Science 260: 1805-1808 (1993); Stahl, et al. J. Biol. Chem. 268: 7628-7631 (1993)]. Thus a soluble LIFR β reagent would only bind to CNTF in the presence of the soluble sR α : $\beta 1$ dimer sCNTFR α : $\beta 1$. For IL-6, the sR α : $\beta 1$ reagent would be IL-6R α : $\beta 1$, and the probe for the $\beta 2$ site would be epitope-tagged sgp130. Thus $\beta 2^-$ mutants of CNTF would be identified as those that bound the sR α : $\beta 1$ reagent, demonstrating that the α and $\beta 1$ site of the cytokine were intact, yet failed to bind the $\beta 2$ reagent.

In addition, the present invention provides for methods of detecting or measuring the activity of potential $\beta 2^-$ mutants by measuring the phosphorylation of a β -receptor component or a signal transduction component selected from the group consisting of Jak1, Jak2 and Tyk2 or any other signal transduction component, such as the CLIPs, that are determined to be phosphorylated in response to a member of the CNTF family of cytokines.

A cell that expresses the signal transduction component(s) described herein may either do so naturally or be genetically engineered to do so. For example, Jak1 and Tyk-2-encoding nucleic acid sequences obtained as described in Velazquez, et al., Cell, Vol. 70:313-322 (1992), may be introduced into a cell by transduction, transfection, microinjection, electroporation, via a transgenic animal, etc., using any known method known in the art.

According to the invention, cells are exposed to a potential antagonist and the tyrosine phosphorylation of either the β -component(s) or the signal transduction component(s) are compared to the tyrosine phosphorylation of

advantage of this screen would be the requirement that the test compounds enter the cell before inhibiting the interaction between the β component and the Jak/Tyk kinase.

The CNTF family antagonists described herein either bind to, or
5 compete with the cytokines CNTF and IL-6. Accordingly, they are useful for treating diseases or disorders mediated by CNTF or IL-6. For example, therapeutic uses of IL-6 antagonists would include the following:

1) In osteoporosis, which can be exacerbated by lowering of estrogen levels in post-menopausal women or through ovariectomy, IL-6 appears to be
10 a critical mediator of osteoclastogenesis, leading to bone resorption [Horowitz, Science 260: 626-627 (1993); Jilka, et al., Science 257: 88-91 (1992)]. Importantly, IL-6 only appears to play a major role in the estrogen-depleted state, and apparently is minimally involved in normal bone maintenance. Consistent with this, experimental evidence indicates that function-blocking antibodies
15 to IL-6 can reduce the number of osteoclasts [Jilka, et al. Science 257: 88-91 (1992)]. While estrogen replacement therapy is also used, there appear to be side effects that may include increased risk of endometrial and breast cancer. Thus, IL-6 antagonists as described herein would be more specific to reduce osteoclastogenesis to normal levels.

20 2) IL-6 appears to be directly involved in multiple myeloma by acting in either an autocrine or paracrine fashion to promote tumor formation [van Oers, et al., Ann Hematol. 66: 219-223 (1993)]. Furthermore, the elevated IL-6 levels create undesirable secondary effects such as bone resorption, hypercalcemia, and cachexia; in limited studies function-blocking antibodies
25 to IL-6 or IL-6Ra have some efficacy [Klein, et al., Blood 78: 1198-1204 (1991); Suzuki, et al., Eur. J. Immunol. 22:1989-1993 (1992)]. Therefore, IL-6 antagonists as described herein would be beneficial for both the secondary

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effects as well as for inhibiting tumor growth.

3) IL-6 may be a mediator of tumor necrosis factor (TNF) that leads to cachexia associated with AIDS and cancer [Strassmann, et al., J. Clin. Invest. 89: 1681-1684 (1992)], perhaps by reducing lipoprotein lipase activity in adipose
5 tissue [Greenberg, et al., Cancer Research 52: 4113-4116 (1992)]. Accordingly, antagonists described herein would be useful in alleviating or reducing cachexia in such patients.

Effective doses useful for treating these or other CNTF family related diseases or disorders may be determined using methods known to one skilled
10 in the art [see, for example, Fingl, et al., The Pharmacological Basis of Therapeutics, Goodman and Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46 ((1975))]. Pharmaceutical compositions for use according to the invention include the antagonists described above in a pharmacologically acceptable liquid, solid or semi-solid carrier, linked to a carrier or targeting
15 molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparation (including antagonist expressing cells) prior to administration *in vivo*. For example, the pharmaceutical composition may comprise one or more of the antagonists in an aqueous solution, such as sterile water, saline, phosphate buffer or
20 dextrose solution. Alternatively, the active agents may be comprised in a solid (e.g. wax) or semi-solid (e.g. gelatinous) formulation that may be implanted into a patient in need of such treatment. The administration route may be any mode of administration known in the art, including but not
25 involved tissue, intraarterially, intranasally, orally, or via an implanted device.

Administration may result in the distribution of the active agent of the

invention throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of agent may be desirable. In some situations, an implant containing active agent may be placed in or near the lesioned area.

- 5 Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

EXAMPLE 1: CNTF COMPETES WITH IL-6 FOR BINDING TO GP130

MATERIALS AND METHODS

- 10 Materials. A clone of PC12 cells that respond to IL-6 (PC12D) was obtained from DNAX. Rat CNTF was prepared as described [Masiakowski, et al., J. Neurochem. 57:1003-10012 (1991)]. IL-6 and sIL-6R α were purchased from R & D Systems. Antisera was raised in rabbits against a peptide derived from a region near the C-terminus of gp130 (sequence:
- 15 CGTEGQVERFETVGME) [SEQ. ID. NO. 2] by the method described (Stahl, et al. J. Biol. Chem. 268:7628-7631 (1993). Anti-phosphotyrosine monoclonal 4G10 was purchased from UBI, and reagents for ECL from Amersham.

- Signal Transduction Assays. Plates (10 cm) of PC12D were starved in serum-free medium (RPMI 1640 + glutamine) for 1 hour, then incubated with
- 20 IL-6 (50 ng/mL) + sIL-6R (1 mg/mL) in the presence or absence of added rat CNTF at the indicated concentrations for 5 minutes at 37°C. Samples were then subjected to anti-gp130 immunoprecipitation, SDS PAGE, and anti-phosphotyrosine immunoblotting as described (Stahl, et al. J. Biol. Chem. 268:7628-7631 (1993).

- 25 RESULTS

The ability of CNTF to block IL-6 responses was measured using a PC12 cell line (called PC12D).that expresses IL-6Ra, gp130, and CNTFR α , but not

LIFR β . As one would predict, these cells respond to IL-6, but not to CNTF (Fig. 2) since LIFR β is a required component for CNTF signal transduction [Davis, et al., Science 260: 59-63 (1993)]. In accordance with results on other cell lines [Ip, et al., Cell 69: 1121-1132 (1992)], PC12D cells give tyrosine

5 phosphorylation of gp130 (as well as a variety of other proteins called CLIPs) in response to 2 nM IL-6 (Fig. 2). Addition of recombinant soluble IL-6R α (sIL-6R α) enhances the level of gp130 tyrosine phosphorylation, as has been reported in some other systems [(Taga, et al., Cell 58: 573-581 (1989))]. However, addition of 2 nM CNTF simultaneously with IL-6 severely
10 diminishes the tyrosine phosphorylation of gp130. Although a slight gp130 phosphorylation response remains in the presence of CNTF, IL-6, and sIL-6R α , it is eliminated if the CNTF concentration is increased fourfold to 8 nM. Thus, in IL-6 responsive cells that contain CNTFR α but no LIFR β , CNTF is a rather potent antagonist of IL-6 action.

15

EXAMPLE 2. BINDING OF CNTF TO THE CNTFR α : β

MATERIALS AND METHODS

20 Scatchard Analysis of CNTF Binding. 125 I-CNTF was prepared and purified as described [Stahl et al. JBC 268: 7628-7631 (1993)]. Saturation binding studies were carried out in PC12 cells, using concentrations of 125 I-CNTF ranging from 20pM to 10nM. Binding was performed directly on a monolayer of cells. Medium was removed from wells and cells were washed once with
25 assay buffer consisting of phosphate buffered saline (PBS; pH 7.4), 0.1mM bacitracin, 1mM PMSF, 1mg/ml leupeptin, and 1mg/ml BSA. Cells were incubated in 125 I-CNTF for 2 hours at room temperature, followed by 2 quick

washes with assay buffer. Cells were lysed with PBS containing 1% SDS and counted in a Packard Gamma Counter at 90-95% efficiency. Non-specific binding was defined by the presence of 100-fold excess of unlabelled CNTF. Specific binding ranged from 70% to 95%.

5 RESULTS

The equilibrium constant for binding of CNTF to CNTFR α : β 1 was estimated from Scatchard analysis of iodinated CNTF binding on PC12D cells (Figure 3). The data is consistent with a 2 site fit having dissociation constants of 9 pM and 3.4 nM. The low affinity site corresponds to interaction of CNTF with CNTFR α , which has a Kd near 3 nM [(Panayotatos, et al., J. Biol. Chem. 268: 19000-19003 (1993)]. We interpret the high affinity complex as the intermediate containing CNTF, CNTFR α , and gp130. A Ewing sarcoma cell line (EW-1) which does contain CNTFR α , gp130, and LIFR β , and therefore gives robust tyrosine phosphorylation in response to CNTF, displays a very similar two site fit with dissociation constants of 1 nM and 10 pM (Wong, et al., unpublished data). Thus it is apparent that CNTF binds with equally high affinity to a complex containing only CNTFR α and gp130, as it does to a complex which additionally contains LIFR β , thus demonstrating the feasibility of creating the sR α : β antagonists described herein.

20

EXAMPLE 3. METHODS OF PRODUCING CYTOKINE LIGAND TRAPS

Virus Stock Production

C20 } ~~SF21 insect cells obtained from *Spodoptera frugiperda* were grown at 27C in Gibco SF900 II medium to a density of 1×10^6 cells/mL. The individual virus stock for either GP130-Fc-His6 (Figure 4) or IL6Ra-Fc (Figure 5) was added to the bioreactor to a low multiplicity 0.01-0.1 PFU/cell to begin the~~

~~infection. The infection process was allowed to continue for 5-7 days allowing maximum virus replication without incurring substantial cell lysis. The cell suspension was aseptically aliquoted into sterile centrifuge bottles and the cells removed by centrifugation. The cell-free supernatant was collected in sterile bottles and stored at 4°C until further use.~~

The virus titer was determined by plaque assay as described by O'Reilly, Miller and Luckow. The method is carried out in 60mm tissue-culture dishes which are seeded with 2×10^6 cells. Serial dilutions of the virus stock are added to the attached cells and the mixture incubated with rocking to allow the virus to adsorb to individual cells. An agar overlay is added and plates incubated for 5 - 7 days at 27C. Staining of viable cells with neutral red revealed circular plaques resulting which were counted to give the virus titer.

Coinfection of Cells for Protein Production

Uninfected SF21 Cells were grown in a 60L ABEC bioreactor containing 40L of SF900 II medium. Temperature was controlled at 27C and the dissolved oxygen level was maintained at 50% of saturation by controlling the flowrate of oxygen in the inlet gas stream. When a density of 2×10^6 cells/mL was reached, the cells were concentrated within the bioreactor to a volume of 20L using a low shear steam sterilizable pump and a with tangential flow filtration device with Millipore Prostack 0.65 micron membranes. After concentration fresh sterile growth medium is slowly added to the bioreactor while the filtration system continues to remove the spent growth medium by diafiltration. After two volume exchanges (40L) have been carried out an additional 20L of fresh medium was added to the bioreactor to resuspend the cells to the original volume of 40L. The cell density was determined once again by counting viable cells using a hemacytometer.

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The required amount of each virus stock was calculated based on the cell density, virus titer and the desired multiplicity of infection (MOI). Virus stock ratios of 5:1, 5:2, 10:2 and 10:4, IL6Ra-Fc to GP130-Fc-His₆ all resulted in production of significant amounts of heterodimer. The ideal virus stock ratio is highly dependent on the ease of purification of the heterodimer from each of the two homodimers. The IL6Ra-Fc homodimer is relatively easy to remove downstream by immobilized metal affinity chromatography. Virus infection ratios have been chosen to minimize the formation of the GP130-Fc-His₆ homodimer which is more difficult to clear downstream. The relative amount of GP130-Fc-His₆ virus stock chosen for infection has increased with successive batches as the purification method for clearing the resultant homodimer has improved.

The virus stocks were aseptically mixed in a single vessel then transferred to the bioreactor. This results in synchronous infection of the SF21 cells. The infection is allowed to proceed for three to four days, allowing sufficient time for maximal production of the heterodimer protein.

Recovery and Protein A Chromatographic Purification

At the conclusion of the infection phase of the bioreactor process the cells were concentrated in the bioreactor using a 10 ft² Millipore Prostak filter (0.65 micron) pore size. The cell-free permeate passing through the filter was collected in a clean process vessel. At the conclusion of the filtration operation the pH of permeate stream, containing the protein product, was adjusted to 8.0 with 10N NaOH. The resultant precipitate was removed by forcing the extract through a 0.8 micron depth filter (Sartorius), followed by a 0.2 micron filter. Sufficient 0.5M EDTA stock was added to give a final concentration of 5mM. The filtered protein solution was loaded onto a 10 cm

diameter column containing 100-200 mL of Pharmacia Protein A Sepharose 4 Fast Flow, equilibrated with PBS. Protein A has a very high affinity for the Fc-Fc domain of each of the 3 recombinant protein products, allowing them to bind while other proteins in the cell-free extract flow through the column.

- 5 After loading the column was washed to baseline with PBS containing an additional 350mM NaCl. The IgG-Fc tagged proteins were eluted at low pH, either with 0.5M acetic acid or with a decreasing pH gradient of 0.1M citric acid and 0.2M disodium phosphate buffers. Tris base or disodium phosphate was added to the eluted protein to avoid prolonged exposure to low pH
- 10 conditions.

- The pooled protein was diafiltered into PBS or HEPES buffer for subsequent and derivitized with 1 mM iodoacetamide to protect the exposed sulfhydryl group on the free cysteine near the hinge region of each Fc domain. This prevents disulfide mediated aggregation of proteins. A 6 ft2
- 15 Millipore spiral wound ultrafiltration membrane with nominal 30 kiloDalton cutoff was used to perform the buffer exchange. The total protein was determined by UV absorbance at 280 nm using the diafiltration buffer as a blank. The relative amounts of heterodimer and two homodimer proteins were determined by SDS PAGE gel electrophoresis using a 6% Tris-Glycine gel
- 20 (Novex). Gels were Coomassie stained then transferred into destain solution overnight. A Shimadzu scanning densitometer was used to determine the relative intensity of the individual protein bands on the SDS PAGE gel. The peak area ratios are used to compute the fraction of heterodimer and each of the homodimers in the column pool fractions.

25

Immobilized Metal Affinity Chromatographic Purification

The six histidine residues on the C-terminus of the GP130-Fc-His6

fusion protein provides an excellent molecular handle for separation of the heterodimeric IL6 antagonist from the two homodimers. The imidazole group on each of the C-terminal histidines of the GP130-Fc-His₆ moiety has a strong binding constant with several divalent metals, including copper, nickel, zinc, cobalt, iron and calcium. Since the IL6Ra-Fc homodimer has no C-terminal histidine residues, it clearly has the lowest affinity. The IL6Ra-Fc-GP130-Fc-His₆ heterodimer has a single strand set six histidines giving it greater affinity for the metal, while the GP130-Fc-His₆ homodimer has two sets of six histidines each giving it the highest affinity of the three IgG tagged proteins to the metal affinity column. Selective elution of the three proteins with increasing amounts of imidazole in the elution buffer therefore elutes the proteins in the following order:

1. IL6Ra-Fc homodimer
2. IL6Ra-Fc-GP130-Fc-His heterodimer
3. GP130-Fc-His homodimer

A 26 mm diameter column containing 100 mL of Pharmacia Chelating Sepharose Fast Flow was saturated with a solution of nickel sulfate until a significant green color is observed in the column eluate. The column is then washed with several column volumes of deionized water, then equilibrated with 50 mM HEPES, 40mM imidazole, pH 8.0. The binding of imidazole to the immobilized nickel results in a green to blue color change. Imidazole was added to the protein load to a final concentration of 40mM. Addition of imidazole to the protein load reduces the binding of IL6Ra-Fc homodimer, increasing the surface area available for the remaining two species. After loading, the column was washed with several column volumes of 50 mM

HEPES, 80mM imidazole, pH 8.0 until a steady baseline was reestablished. The heterodimer was selectively eluted with 50 mM HEPES, 150mM imidazole, pH 8.0 over several column volumes. The protein fractions were pooled and diafiltered into PBS as described in the section above.

5

EXAMPLE 4. ALTERNATIVE METHODS OF CONSTRUCTING LIGAND TRAPS

BACKGROUND

10 As described above, receptor activation by CNTF, and analogously by IL-6 and IL-11, follows an ordered sequence of binding events (Figure 6). The cytokine initially binds to its cognate $R\alpha$ with low affinity ($K_d = 3$ to 10 nM); this is a required step - cells which do not express the cognate $R\alpha$ do not respond to the cognate cytokine. The cytokine• $R\alpha$ complex associates with
15 the first signal transducing component, gp130, to form a high affinity complex (K_d in the order of 10 pM for the CNTF•CNTF $R\alpha$ •gp130 complex). This complex does not transduce signal, as it is the dimerization of the signal transducing components that brings about signaling (Stahl and Yancopoulos, J. Neurobiology 25: 1454-1466 (1994); Stahl et al., Science 267:1349-1353 (1995);
20 Davis et al., Science 260:1805-1808 (1993); Stahl et al., Science 263:92-95 (1994); Murakami, et al. Science 260:1808-1810 (1993). At least in the case of IL-6, the cytokine• $R\alpha$ •signal transducer heterotrimeric complex subsequently associates with another like complex, to form a hexameric complex (Figure 6) (Ward et al., J. Biol. Chem. 269:23286-23289 (1994). The resulting dimerization
25 of the signal transducers - gp130 in the case of IL-6 (Murakami et al., Science 260:1808-1810 (1993) and IL-11, gp130 and LIFR in the case of CNTF (Davis et al., Science 260:1805-1808 (1993) - brings about signal transduction.

The initial heterodimeric molecules made comprised a soluble $R\alpha$ -component linked to the extracellular domain of gp130. These molecules

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were shown to mimic the high affinity cytokine•Rα•gp130 complex and behave as a high affinity antagonist of their cognate cytokine (Figure 7). To make these molecules, the extracellular domain of gp130 was paired with the extracellular domain of the α-receptor components for IL-6 and CNTF, IL-6Rα and CNTFRα respectively. To link the Rα with the extracellular domain of gp130, the soluble Rα-components and gp130 were fused to the Fc portion of human IgG1 to produce Rα-Fc and gp130-Fc respectively. The Fc domain was chosen primarily but not solely because it naturally forms disulfide-linked dimers. Heterodimeric molecules comprising Rα-Fc•gp130-Fc were expressed, purified and shown to behave as highly potent antagonists of their cognate ligand. Furthermore, these molecules were found to be highly specific for their cognate cytokine since it is the choice of the α-receptor component which specifies which cytokine is bound and trapped (there is no measurable binding of the cytokine to gp130 in the absence of the appropriate Rα).

Here we describe an extension of this technology which allows the engineering of different heteromeric soluble receptor ligand traps which by virtue of their design may have additional beneficial characteristics such as stability, Fc-receptor-mediated clearance, or reduced effector functions (such as complement fixation). Furthermore, the technology described should prove suitable for the engineering of any heteromeric protein in mammalian or other suitable protein expression systems, including but not limited to heteromeric molecules which employ receptors, ligands, and catalytic components such as enzymes or catalytic antibodies.

MATERIALS AND METHODS

Genetic Engineering of heteromeric immunoglobulin heavy/light chain soluble receptor-based ligand traps for IL-6.

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The IL-6 traps described here were engineered using human gp130, human IL-6 α -receptor (IL-6R α), the constant region of the heavy chains (C γ) of human IgG1 (C γ 1) (Lewis et al., Journal of Immunology 151:2829-2838 (1993) or IgG4 (C γ 4) with or without a join-region (J), and the constant regions of kappa (κ) and lambda (λ) (Cheung, et al., Journal of Virology 66:6714-6720 (1992) light chains of human immunoglobulin (Ig), also with or without a different j-peptide (j). This design takes advantage of the natural ability of the C γ domain to heterodimerize with κ or λ light chains. The heterodimerization of C γ with the light chain occurs between the CH1 domain of C γ and the constant region of the light chain (C L), and is stabilized by covalent linking of the two domains via a single disulfide bridge. We reasoned that, like the Fc domain of human IgG1, the combination of C γ with C L could be used to produce disulfide linked heteromeric proteins comprised of the extracellular domain of gp130 on one chain and the extracellular domain of IL-6R α on the other chain. Like their Fc-based counterparts, such proteins were postulated to be high affinity ligand traps for IL-6 and as a result to inhibit the interaction of IL-6 with the native receptor on IL-6-responsive cells, thus functioning as IL-6 antagonists. Furthermore, constructs employing the full length C γ region would, much like antibodies, form homodimers of the C γ chain, giving rise to antibody-like molecules comprising of two "light chains" and two "heavy chains" (figure 8). The potential advantage of this design is that it may more closely mimic the IL-6•IL-6R α •gp130 complex and may display a higher affinity for the ligand than comparable single heterodimers. An additional design is incorporated by using truncated versions of C γ , comprised only of the CH1 domain. These will form heterodimeric molecules with receptor- κ fusion proteins, and will thus resemble the Fab fragment of antibodies.

C21

5 ~~All the soluble receptor Ig chimeric genes may be engineered in~~
plasmid vectors including, but not limited to, vectors suitable for
mammalian expression (Cos monkey kidney cells, Chinese Hamster Ovary
cells [CHO], and ras-transformed fibroblasts [MG-ras]) and include a Kozak
sequence (CGC CGC CAC CAT GGT G) at the beginning of each chimeric gene
for efficient translation. Engineering was performed using standard genetic
engineering methodology. Each construct was verified by DNA sequencing,
mammalian expression followed by western blotting with suitable antibodies,
biophysical assays that determine ligand binding and dissociation, and by
10 growth inhibition assays (XG-1, as described later). Since the domains utilized
to engineer these chimeric proteins are flanked by appropriate restriction
sites, it is possible to use these domains to engineer other chimeric proteins,
including chimeras employing the extracellular domains of the receptors for
factors such as IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, LIF, IL-11, IL-15, IFN γ , TGF β ,
15 and others. The amino acid coordinates for each component utilized in
making the IL-6 traps are listed below (Note: numbering starts with the
initiating methionine as #1; long sequences are listed using the single letter
code for the twenty amino acids):

(a) Constructs employing human gp130:

- 20 (i) **gp130-Cy1** was engineered by fusing in frame the extracellular domain of
gp130 (amino acids 1 to 619) to a Ser-Gly bridge, followed by the 330 amino
acids which comprise Cy1 and a termination codon (Figure 9).
- (ii) **gp130-J-Cy1** was engineered in the same manner as gp130-Cy1 except that a
J-peptide (amino acid sequence: GQGTLVTVSS) was inserted between the Ser-
25 Gly bridge and the sequence of Cy1 (see Figure 9).
- (iii) **gp130 Δ 3fibro-Cy1** was engineered by fusing in frame the extracellular
domain of gp130 without its three fibronectin-like domains (Figure 10). The

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Cont

remaining part of this chimeric protein is identical to gp130-C γ 1.

(iv) **gp130-J-CH1** was engineered in a manner identical for that described for gp130-C γ 1, except that in place of the C γ 1 region only the CH1 part of C γ 1 has been used (Figure 11). The C-terminal domain of this construct includes the part of the hinge that contains the cysteine residue responsible for heterodimerization of the heavy chain of IgG with a light chain. The part of the hinge that contains the two cysteines involved in C γ 1 homodimerization has been deleted along with the CH2 and CH3 domains.

(v) **gp130-C γ 4** was engineered in a manner identical to that described for gp130-C γ 1, except that C γ 4 was used in place of C γ 1 (Figure 12). In addition, an *Rsr*II DNA restriction site was engineered at the hinge region of the C γ 4 domain by introducing two silent base mutations. The *Rsr*sII site allows for other desired genetic engineering manipulations, such as the construction of the CH1 equivalent of gp130-C γ 4.

(vi) **gp130- κ** was engineered in a manner identical to that described for gp130-C γ 1, except that the constant region of the κ light chain of human Ig was used in place of C γ 1 (Figure 13).

(vi) **gp130-J- κ** was engineered in a manner identical to that described for gp130-J- κ , except that a j-peptide (amino acid sequence: TFGQGTKVEIK) was inserted between the Ser-Gly bridge and the κ -region.

(viii) **gp130- λ** was engineered in a manner identical to that described for gp130-C γ 1, except that the constant region of the λ light chain (Cheung, et al., Journal of Virology 66:6714-6720 (1992) of human Ig was used in place of C γ 1 (Figure 14).

Constructs employing human IL-6Ra:

(i) **IL6R-C γ 1** was engineered by fusing in frame amino acids 1 to 358 of IL-6R α (Yamasaki et al., Science 241:825-828 (1988), which comprise the extracellular

domain of IL-6R α (Figure 15), to an Ala-Gly bridge, followed by the 330 amino acids which comprise Cy1 and a termination codon.

(ii) **IL6R-κ** was engineered as described for IL6R-Cγ1, except that the κ-domain (Figure 13) utilized for gp130-κ was used in place of Cγ1.

5 (iii) **IL6R-j-κ** was engineered as described for IL6R-κ except that the j-peptide described for gp130-j-κ was placed between the Ala-Gly bridge and the κ-domain.

(iv) Three additional constructs, IL6R313-C γ 1, IL6R313- κ , and IL6R313-j- κ , were engineered as using a truncated form of IL-6Ra comprised of amino acids 1 to 313 (Figure 16). Each of these constructs were made by fusing in frame IL6R313 with a Thr-Gly bridge followed by the C γ 1, κ -, and j- κ -domains described above. These constructs were engineered in order to complement the gp130 Δ 3fibro-derived constructs.

Expression and purification of ligand traps

15 To produce covalently linked heterodimers of soluble gp130 and
soluble IL-6R α , gp130-Ig chimeric proteins were co-expressed with appropriate
IL-6R α -Ig chimeric proteins in complementing pairs. Co-expression was
achieved by co-transfecting the corresponding expression vectors into suitable
mammalian cell lines, either stably or transiently. The resulting disulfide-
20 linked heterodimers were purified from conditioned media by several
different methods, including but not limited to affinity chromatography on
immobilized Protein A or Protein G, ligand-based affinity chromatography,
ion exchange, and gel filtration.

25 An example of the type of methods used for purification of a heavy/light receptor fusion protein is as follows: gp130-Cy1•IL-6R α - κ was expressed in COS cells by co-transfecting two different vectors, encoding gp130-Cy1 and IL-6R α - κ respectively. Serum-free conditioned media (400 ml) were collected two days post-transfection and Cy1-

bearing proteins were purified by affinity chromatography over a 1ml Protein A Sepharose (Pharmacia). The material generated in this step was further purified by a second affinity chromatography step over a 1 ml NHS-activated Sepharose (Pharmacia) which was derivatized with recombinant human IL-6, in order to remove gp130-Cy1 dimer from gp130-Cy1•IL-6R α - κ complexes (the gp130-Cy1 dimer does not bind IL-6). Proteins generated by this method were more than 90% pure, as evidenced by SDS-PAGE followed by silver-staining (Figure 17). Similar protocols have been employed successfully towards the purification of other heavy/light receptor heterodimers.

RESULTS

Biological activity of immunoglobulin heavy/light chain receptor fusion antagonists

The purified ligand traps were tested for their ability to bind IL-6 in a variety of different assays. For example, the dissociation rate of IL-6 bound to the ligand trap was measured in parallel with the dissociation rate of IL-6 from the anti-IL-6 monoclonal neutralizing antibody B-E8 [Brochier, et al., Int. J. Immunopharmacology 17:41-48 (1995), and references within]. An example of this type of experiment is shown In Figure 18. In this experiment 20 pM 125 I-IL-6 (1000 Ci/mmol; Amersham) was preincubated with 500 pM of either gp130-Cy1•IL-6R α - κ or mAb B-E8 for 20 hours. At this point a 1000-fold excess (20 nM) of "cold" IL-6 was added. Periodically, aliquots of the reaction were removed, the ligand trap or B-E8 were precipitated with Protein G-Sepharose, and the number of cpm of 125 I-IL-6 that remained bound was determined. Clearly, the dissociation rate of human 125 I-IL6 from the ligand trap was very slow - after three days, approximately 75% of the initial counts were still bound to the ligand trap. In contrast, less than 5% of the counts remained associated with the antibody after three days. This result demonstrates that the dissociation rate of the ligand from these ligand traps is very slow.

(22) ~~In a different set of experiments the ability of the ligand traps to~~

dependent manner (Figure 20). Of the five different traps tested gp130-C γ 1•IL-6R α - κ was the most active and essentially display the same neutralizing activity towards IL-6 as the antibody B-E8. As little as a 10-fold molar excess of either gp130-C γ 1•IL-6R α - κ or B-E8 completely blocked the activity of IL-6 (a reading of A570-650 = 0.3 AU corresponds to no proliferation of the XG-1 cells). At a 100-fold molar excess all of the ligand traps tested completely blocked the activity of IL-6. This observed inhibition is highly selective as neither a gp130-Fc•CNTFR α -Fc ligand trap which blocks CNTF activity, nor gp130-Fc homodimer exhibit any blocking activity towards IL-6 even when used at a 1000-fold molar excess over IL-6 (data not shown). This data demonstrates that the heteromeric immunoglobulin heavy/light chain receptor-based ligand traps function as selective high affinity antagonists of their cognate ligand.

15 EXAMPLE 5 - CLONING OF FUSION POLYPEPTIDE COMPONENTS

The extracellular domains of the human cytokine receptors were obtained by standard PCR techniques using tissue cDNAs (CLONTECH), cloned into the expression vector, pMT21 (Genetics Institute, Inc.), and the sequences were sequenced by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). For the IL-4Ra, nucleotides 241 through 868 (corresponding to the amino acids 24-231) from the Genbank sequence, X52425, were cloned. For the IL-2R γ , nucleotides 15 through 776 (corresponding to amino acids 1-233) from the Genbank sequence, D11086, were cloned. For the IL-6Ra, nucleotides 52 through 1044 (corresponding to the amino acids 1-331) from the Genbank sequence, X52425, were cloned. For gp130, nucleotides 322 through 2112 (corresponding to the amino acids 30-619) from the Genbank sequence, M57230, were cloned. For the IL-1RAcP, nucleotides 1 through 1074

(corresponding to the amino acids 1-358) from the Genbank sequence, AB006357, were cloned. For the IL-1RI, nucleotides 55 through 999 (corresponding to the amino acids 19-333) from the Genbank sequence, X16896, were cloned.

5

EXAMPLE 6 - PRODUCTION OF FUSION POLYPEPTIDES (CYTOKINE TRAPS)

C23/ 10 ~~The nucleotide sequences encoding the cytokine traps were constructed from the individual cloned DNAs (described *supra*) by standard cloning and PCR techniques. In each case, the sequences were constructed in frame such that the sequence encoding the first fusion polypeptide component was fused to the sequence encoding the second fusion polypeptide component followed by~~
15 ~~an Fc domain (hinge, CH2 and CH3 region of human IgG1) as the multimerizing component. In some cases extra nucleotides were inserted in frame between sequences encoding the first and second fusion polypeptide components to add a linker region between the two components (See Figs. 21A-21D - trap 424; Figs. 24A-24F - trap 412; and Figs. 26A-26E - trap 569).~~

C24/ 20 ~~For the IL-4 traps, 424 (Figs. 21A-21D), 603 (Figs. 22A-22D) and 622 (Figs. 23A-23D), the IL-2R γ component is 5', followed by the IL4R α component and then the Fc component. For the IL-6 traps, 412 (Figs. 24A-24F) and 616 (Figs. 25A-25F), the IL-6R α component is 5' followed by the gp130 component and then~~
25 ~~the Fc domain. For the IL-1 trap 569 (Figs. 26A-26E), the IL-1RAcP component is 5' followed by the IL-1RI component and then the Fc domain. The final constructs were cloned into the mammalian expression vector pCDNA3.1 (STRATAGENE).~~

C26

In the 569 sequence (Figs. 26A-26E), nucleotides 1-1074 encode the IL1RAcP component, nucleotides 1075-1098 encode a linker region, nucleotides 1099-2043 encode the IL1RI component and nucleotides 2044-2730 encode the Fc domain.

5

C26

In the 412 sequence (Figs. 24A-24F), nucleotides 1-993 encode the IL6R α component, nucleotides 994-1023 encode a linker region, nucleotides 1024-2814 encode the gp130 component and nucleotides 2815-3504 encode the Fc domain.

10

C27

In the 616 sequence (Figs. 25A-25F), nucleotides 1-993 encode the IL6R α component, nucleotides 994-2784 encode the gp130 component and nucleotides 2785-3474 encode the Fc domain.

15

C28

In the 424 (Figs. 21A-21D) and 622 (Figs. 23A-23D) sequences, nucleotides 1-762 encode the IL2R γ component, nucleotides 763-771 encode a linker region, nucleotides 772-1395 encode the IL4R α component and nucleotides 1396-2082 encode the Fc domain.

20

C29

Finally, in the 603 sequence (Figs. 22A-22D), nucleotides 1-762 encode the IL2R γ component, nucleotides 763-1386 encode the IL4R α component and nucleotides 1387-2073 encode the Fc domain.

25

DNA constructs were either transiently transfected into COS cells or stably transfected into CHO cells by standard techniques well known to one of skill in the art. Supernatants were collected and purified by protein A affinity chromatography and size exclusion chromatography by standard techniques. (See for example Harlow and Lane, Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

Example 7: IL-4 Bioassay Protocol using TF-1 (ATCC) cells.

5

Reagents and Equipment Needed

MTT Dye Solution:

MTT(3-[4,5-Dimethylthiazole-2-yl]) (Sigma catalog# M2128)

- 10 Working concentration: Dissolve 5 mg of anhydrous MTT in 200 ml PBS without Ca^{+2} , Mg^{+2} .

Sterile filter and store aliquoted at -20°C

Solubilization Solution:

- 15 For 1000 ml, combine 100 g SDS, 950 ml dH_2O , 50 ml Dimethyl Formamide, and 850 μl concentrated HCl.

Filter sterilize with a $0.45\mu\text{m}$ filter unit.

Store at room temperature

- 20 TF-1 cell Growth Medium: RPMI 1640, 10%FBS, Pen/Strep, 2mM L-glutamine

- Other: 0.4% Trypan Blue Stain, sterile tubes for dilutions, sterile 96 well cell culture plates (Falcon#3072), hemacytometer, centrifuge, ELISA plate reader,
25 multichannel pipet for 15, 25, 50 and $100\mu\text{l}$ volume, sterile reagent reservoirs, sterile pipet tips, gloves.

Assay Protocol

- 30 A. Preparation of Assay plates

1. Prepare sterile 96 well tissue culture plates to contain $50\mu\text{l}$ of growth medium per well with various concentrations of IL-4 and 10nM IL-4 antagonist. This can be done by preparing a working dilution of IL-4 that is 4
35 times the highest concentration to be assayed. In separate tubes, do a two-fold

serial dilution of the IL-4. Add 25µl of each dilution to one row across the plate (i.e. row A gets highest concentration, row G gets lowest concentration). Add 25µl of growth medium without IL-4 to row H. Prepare the antagonists to be tested by making a stock that is 4 times the final concentration. Add
5 25µl to a triplicate set of IL-4 containing wells (columns 1,2,3, A through H). Be sure to include antagonist in row H.

2. As a positive control, leave one set with no antagonist. These wells will contain IL-4 and media only.

10

3. Incubate the plate for 1-2 hours at 37°C in a humidified 5% CO₂ incubator before preparing cells to be used for assay.

B. Preparation of Cells

15

4. Wash cells twice by centrifugation in assay medium free of growth factor.

5. Determine cell number and trypan blue viability and suspend cells to a final concentration of 8×10^5 /ml in assay medium.

20

6. Dispense 50µl of the cell suspension (40,000 cells) into all wells of the plates. Total volume should now be 100µl/well.

7. Incubate the plate at 37°C for 68 hours in a humidified 5% CO₂ incubator.

25

C. Color Development

8. After incubating for 68 hours, add 15µl of the MTT dye solution to each well.

30

9. Incubate the plate at 37°C for 4 hours in a humidified 5% CO₂ incubator.

10. After 4 hours, add 100µl of the solubilization solution to each well. Allow the plate to stand overnight in a sealed container to completely solubilize the formazan crystals.

35

11. Record the absorbance at 570/650nm.

Results:

5

Figure 27 shows that an IL-4 trap designated 4SC375, which is a fusion polypeptide of IL-2R γ -scb-IL4R α -Fc Δ C1, is several orders of magnitude better as an IL-4 antagonist than IL4R α Fc Δ C1 alone in the TF1 cell bioassay.

10

Figure 28 shows that the IL-4 trap designated 4SC375 shows antagonistic activity in the TF1 cell bioassay equivalent to an IL-4 trap designated 4SC424 which is a fusion polypeptide of IL-2R γ -IL4R α -Fc Δ C1 having the IL-2R γ component flush with the IL-4R α component.

15

Example 8: IL-6 Bioassay Protocol using XG-1 cells

Reagents and Equipment Needed

20

MTT Dye Solution:

MTT(3-[4,5-Dimethylthiazole-2-yl]) (Sigma catalog# M2128)

Working concentration: Dissolve 5 mg of anhydrous MTT in 200 ml PBS without Ca⁺², Mg⁺².

Sterile filter and store aliquoted at -20°C

25

Solubilization Solution:

For 1000 ml, combine 100 g SDS, 950 ml dH₂O, 50 ml Dimethyl Formamide, and 850 μ l concentrated HCl.

Filter sterilize with at 0.45 μ m filter unit.

30

Store at room temperature

Assay Medium: RPMI

1640, 10%FBS, Pen/Strep, 2mM L-glutamine, 50 μ M mercapto-ethanol.

Other: 0.4% Trypan Blue Stain, sterile tubes for dilutions, sterile 96 well cell culture plates (Falcon#3072), hemacytometer, centrifuge, ELISA plate reader, multichannel pipet for 15, 25, 50 and 100µl volume, sterile reagent reservoirs, sterile pipet tips, gloves.

Assay Protocol

A. Preparation of Assay plates

1. Prepare sterile 96 well tissue culture plates to contain 50µl of growth medium per well with various concentrations of IL-6 and 10nM IL-6 antagonist. This can be done by preparing a working dilution of IL-6 that is 4 times the highest concentration to be assayed. In separate tubes, do a two-fold serial dilution of the IL-6. Add 25µl of each dilution to one row across the plate (i.e. row A gets highest concentration, row G gets lowest concentration). Add 25µl of growth medium without IL-6 to row H. Prepare the antagonists to be tested by making a stock that is 4 times the final concentration. Add 25µl to a triplicate set of IL-6 containing wells (columns 1,2,3, A through H). Be sure to include antagonist in row H. A typical IL-6 titration starts at 200ng/ml down to 3.1ng/ml.

2. As a positive control, leave one set with no antagonist. These wells contain IL-6 and media in place of antagonist.

3. Incubate the plate 1-2 hours at 37°C in a humidified 5% CO₂ incubator before preparing cells to be used for assay.

B. Preparation of Cells

4. Wash cells twice by centrifugation (5 min at 1000RPM) in assay medium free of growth factor.

5. Determine cell number and trypan blue viability and suspend cells to a final concentration of 8×10^5 /ml in assay medium.

6. Dispense 50µl of the cell suspension (40000 cells) into all wells of the plates. Total volume should now be 100µl/well.

7. Incubate the plate at 37°C for 68 hours in a humidified 5% CO₂ incubator.

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C. Color Development

8. At 68 hours add 15µl of the dye solution to each well.

10 9. Incubate the plate at 37°C for 4 hours in a humidified 5% CO₂ incubator.

10. After 4 hours, add 100µl of the solubilization solution to each well. Allow the plate to stand overnight in a sealed container to completely solubilize the formazan crystals.

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11. Record the absorbance at 570/650nm.

Results:

C30 20 ~~Figure 29 shows that the IL 6 trap (6SC412 IL 6R-sch gp-FcΔC1) described in Figs. 24A-24F is a better antagonist of IL-6 in the XG1 bioassay than the neutralizing monoclonal antibody to human IL-6 - BE8.~~

25 Example 9: MRC5 Bioassay for IL1 Traps

C31 MRC5 human lung fibroblast cells respond to IL-1 by secreting IL-6 and thus were utilized to assay the ability of IL-1 traps to block the IL-1-dependent production of IL-6. IL1 Trap 1SC569 (Figs. 26A-26E) was tested against IL-1-RI.Fc which is the extracellular domain of the IL-1 Type I receptor fused to an Fc domain.

MRC5 cells are suspended at 1×10^5 cells per ml in medium and 0.1 ml of cells are plated (10,000 cells per well) into the wells of a 96 well tissue culture plate. Plates are incubated for 24 hours at 37°C in a humidified 5% CO₂ incubator.

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IL-1 trap and recombinant human IL-1 at varying doses are pre-incubated in a 96 well tissue culture dish and incubated for 2 hours at 37°C. 0.1 ml of this mixture is then added to the 96 well plate containing the MRC5 cells such that the final concentration of IL-1 Trap is 10nM and the final concentrations of the IL-1 ranges from 2.4 pM to 5nM. Control wells contain trap alone or nothing.

Plates are then incubated at 37°C for 24 hours in a humidified 5% CO₂ incubator. Supernatant is collected and assayed for levels of IL-6 using R&D Systems Quantikine Immunoassay Kit according to the manufacturer's instructions.

Results:

C32 Figure 30 shows that the trap 569 (Figs. 26A-26E) is able to antagonize the effects of IL-1 and block the IL-6 production from MRC 5 cells upon treatment with IL-1. At a concentration of 10nM, the trap 569 is able to block the production of IL-6 up to an IL-1 concentration of 3nM. In contrast, the IL-1RI.Fc is a much poorer antagonist of IL-1. It is only able to block the effects of IL-1 up to about 10-20 pM. Thus, the trap 569 is approximately 100x better at blocking IL-1 than IL1RI.Fc.

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Example 10 - Construction of IL-13/IL-4 single chain traps

1. PCR cloning of human IL-13R α 1 from human spleen cDNA.

- The extracellular domain of human IL-13R α 1 (corresponding to
5 nucleotides #34-1062 from Genbank Accession # Y09328) was amplified by
standard PCR techniques. The PCR product was then ligated into an
expression vector (pCDNA3.1 Stratagene) containing human Fc construct to
create a fusion protein consisting of the extracellular domain of human IL-
13R α 1 fused in frame with the hinge, CH2 and CH3 region of human IgG1.
10 The human IL-13R α 1 sequence was verified. The DNA encoding IL-13R α 1.Fc
was transfected into COS cells and protein expression was verified by Western
Blot analysis of the cell media.

15 2. Construction of IL-13/IL-4 Traps

a. IL-4R α .IL-13R α 1.Fc

- The IL-13R α 1 extracellular domain (not including the predicted signal
peptide, therefore nt #100-1062; amino acid #23-343) was amplified by
standard PCR techniques and ligated into an expression vector, pMT21, to
20 create an in frame fusion downstream of the extracellular domain of human
IL-4R α (nucleotides 176 through 868 (corresponding to the amino acids 1-231)
from the Genbank sequence X52425) and upstream of the human Fc. The IL-
4R α contains a nucleotide change at nt# 794 (T to A) to change the amino acid
at #207 to a Ser from a Cys. A two amino acid linker with the amino acid
25 sequence SG was constructed in frame between the IL-4R α and the IL-13R α 1.
The human IL-13R α 1 sequence was verified. The construct encoding this
single chain trap was transfected into COS cells and protein expression was
verified by Western Blot analysis of the cell media. The nucleotide and

amino acid sequence of the IL-4R α .IL-13R α 1.Fc single chain trap is set forth in Figure 31A-31G.

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~~b. IL-13R α 1-IL-4R α .Fc~~

5 The IL-13R α 1 extracellular domain (including the signal peptide, therefore
nts #34-1062; amino acid #1-343) was amplified by standard PCR techniques
and ligated into an expression vector, pJFE14, to create an in-frame fusion
upstream of the human IL-4Ra (nucleotides 236 through 868 (corresponding
to the amino acids 21-231) from the Genbank sequence X52425) and human
10 Fc. A ten amino acid linker with the amino acid sequence GAPSGGGGRP
was constructed in frame between the IL-4R α and the IL-13R α 1. The human
IL-13R α 1 sequence was verified. The construct encoding this single chain trap
was transfected into COS cells and protein expression was verified by Western
Blot analysis of the cell media. The nucleotide and amino acid sequence of the
15 ~~IL-13R α 1-IL-4R α .Fc single chain trap is set forth in Figure 32A-32G.~~

The present invention is not to be limited in scope by the specific
embodiments described herein. Indeed, various modifications of the
20 invention in addition to those described herein will become apparent to those
skilled in the art from the foregoing description and accompanying figures.
Such modifications are intended to fall within the scope of the appended
claims.